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13. ABSTRACT *(Maximum 200 Words)*

The original goal was to purify both NK cell receptor molecules (Ly49C) and cognate class I MHC ligands (H2-Kb) to determine the crystal structure of Ly49C bound to Kb. This aim has been achieved by others, using Ly49A and its ligand H2-Dd. The 'missing self' hypothesis is supported by many recent studies. The current goal is to use this knowledge to devise anti-tumor treatments. According to the hypothesis, exposure of NK cell receptors to 'self' class I antigens induces a signal that inactivates the lytic 'positive signal' (by activating phosphatases that dephosphorylate signal molecules). If we treat mice bearing C1498 leukemia cells with non-depleting antibody fragments to NK inhibitory receptors, survival is enhanced. NK cells treated with F(ab')2 blocking antibodies 'purge' leukemic cells from marrow cell suspensions to enhance survival. The aims to pursue preliminary findings are 1] to determine how the tumor cells are killed (apoptosis or necrosis), 2] to develop rapid assays for tumor presence prior to overt disease to improve the ability to evaluate treatment, and 3] extend anti-tumor studies to include use of multiple anti-receptor antibodies, and to use adoptive transfer of activated NK cells 'coated' with blocking antibodies to augment therapy.

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Introduction

Natural killer cells lyse tumor cells, virally infected cells, autologous T cells that lose expression of class I antigens, and even autologous dendritic cells that express normal levels of class I antigens(1-3). Based on the findings that F1 hybrid mice reject parental strain marrow cell grafts (4) and that class I low tumor cells are preferentially susceptible to NK cell lysis, Ljunggren and Kaerre developed the "missing self' hypothesis (5). They predicted that some NK cell receptor would recognize a ligand on prospective target cells that would prevent rather than induce a signal to kill the cell. Yokoyama discovered the Ly49 genes and described the NK gene complex on chromosome 6 of mice (6,7). These genes encoded lectin-like receptors that were homodimers and recognized class I molecules. Initially only inhibitory receptors were detected, but eventually positive signaling receptors were discovered that lack the inhibitory motifs in the cytoplasmic domains. Our group presented evidence that NK cell lysis of tumor cells in vitro and rejection of marrow grafts in vivo were governed by the presence of activating Ly49D receptors and presence or absence of inhibitory Ly49G2, A or C receptors (8, 9). These and other data led to the hypothesis that NK cell anti-syngeneic (autologous) leukemia effects should be greatly enhanced if we could block negative signals to class I antigens. Antibodies to the class I antigens or to the Ly49 receptors do enhance lysis in vitro (10). However, because so many cells express class I in the body, we chose to use the monoclonal antibodies (mAb) to Ly49 inhibitory receptors. If we treat C57BL/6 (B6) mice bearing syngeneic C1498 leukemia cells with non-depleting antibody $F(ab')_2$ fragments to NK Ly49I and C inhibitory receptors, survival is enhanced. This reagent allows B6 NK cells stimulated with IL-2 to 'purge' leukemia cells from marrow cell suspensions. The transfer of these purged marrow cells to irradiate mice enhances survival relative to the transfer of similar cells without treatment with the blocking reagent. To pursue these preliminary findings we plan to use improved reagents, determine how the tumor cells die in vivo (apoptosis or necrosis), and extend anti-tumor studies by adoptive transfer of activated NK cells 'coated' with blocking antibodies in mice bearing C1498 leukemia cells.

Body

1. Blocking negative signals to Ly49I and Ly49C receptors with 5E6 F(ab')2 mAbs augments lysis of syngeneic tumor cells in vitro (Figure 1). Representative data are presented to indicate that this non-depleting mAb fragment augments lysis of C1498 or EL-4 B6 tumor cells by purified $NK1.1^{+}5E6^{+}$ NK cells in vitro. This type of result has been observed by many investigators and support the missing self concept. The staining of NK1.1+ NK cells with whole or F(ab')2 5E6 mAbs is also presented.

2. Blocking negative signals to Ly49I and Ly49C receptors with 5E6 F(ab')2 mAbs augments the ability of NK cells to inhibit tumor cell colony formation in vitro. In this representative experiment, IL-2 activated B6 NK cells were cultured for 3 hours in the presence of medium, control IgG, F(ab')2 4D11 anti-Ly49G2, or $F(ab')_2$ 5E6 anti-Ly49I & Ly49C mAbs for 3 hours. Ly49G2 is an inhibitory receptor that recognizes H2-D^d and Ly49C and Ly49I recognize H2-K^b and Ly49C also recognizes H2-D^d. Following this incubation, B6 C1498 or EL-4, or DBA/2 P815 tumors cells were cultured with the cells for 48 hours. The cells were then placed at low cell numbers in semi-solid matrix culture for 6 days. The data in Figure 2 shows that 5E6 $F(ab')_2$

augmented the ability of B6 NK cells to suppress tumor colony-formation of all 3 tumor types, while 4D11 $F(ab')_2$ only enhanced the ability of B6 NK cells to inhibit P815 colony formation. These promising results justified extension of these findings to in vivo tumor studies.

3. Blocking negative signals to $5E6^+$ NK cells results in anti-C1498 leukemia in vivo. In the first set of experiments, B6 mice were each infused i.v. with a lethal inoculum of 3×10^4 C1498 cells. The mice (groups of 8) were injected i.p. with 180 mg 5E6 $F(ab')_2$ mAbs 2 days before challenge and 3 twice a week for 3 weeks after challenge. The control mice injected with only vehicle died were all dead by 31 days (Figure 3a). The mice treated with the blocking reagent prolonged survival in 3/4 of the mice and 1/4 of the mice survived. Three other experiments gave similar results. The limitation of this treatment includes the short half-life of $F(ab')_2$ mAbs (circa 18 hours). We next tested the effect of 'purging' C1498 cells in vitro and infusion of NK cells coated with F(ab')2 5E6 mAbs at a later time (Fig. 3b). Twelve million IL-2 activated B6 NK cells were incubated with 1.2 million C1498 leukemia cells in the presence of normal mouse serum (NMS), or 5E6 or 4D11 $F(ab')_2$ mAbs for 24 hours. Inocula of 10^5 cultured C1498 cells (along with the NK cells) were infused into each B6 mouse. At 18 and 25 days later groups of mice were infused with 5 million B6 NK cells pretreated with F(ab')2 5E6 or 4D11, or NMS and were injected with 5×10^4 units of hIL-2 to stimulate the NK cells. This approach proved to be an improvement, although it is admittedly somewhat artificial. The group of 20 mice that received C1498 cells and $F(ab')_2$ mAb treated NK cells survived longer and 1/2 lived >80 days. Control mice died in about 32 days. The NK cells treated with NMS or 4D11 $F(ab')_2$ mAb survived longer than controls but none survived > 62 days. The p values calculated by the Long-rank test were highly significant.

4. Development of imaging methods to analyze anti-leukemia effects in vivo. The Radiological Science group at this University is developing imaging methods that are adapted to use of small experimental animals. We want to use imaging methods that would allow us to visualize rapid assays for anti-tumor effects and to detect mechanisms of cell death of the targeted tumor cells. The goal is to develop rapid assays that will analyze the ability of these blocking antibodies to augment anti-leukemia effects as early as possible after infusion of the leukemia cells. This includes the very early step of clearance of radiolabeled tumor cells from the lung (11). In figure 4 we show images of a mouse infused with ^{111}In-labeled YAC-1 tumor cells that are very sensitive to NK cell lysis. At 15 min. localization is restricted to the lungs. By 3 hours, the lungs contain much less of the ^{111}In, while the liver/spleen area has now gained radioactivity. In addition, a ^{99m}Tc agent that goes to liver/spleen but not lung, was infused just before the 3 hour point. The overlay of the two isotopes (green and red) produced a yellow area over the liver/spleen (the bright area in this black and white production). Counts of the lung, spleen and liver removed form the animal after sacrifice mirrored the data obtained by imaging. This method could be used to test for relative rate of tumor cell killing in mice injected with blocking antibodies. The imaging method can also be used to detect apoptosis using radio-labeled molecules that detect cells undergoing apoptosis versus necrosis.

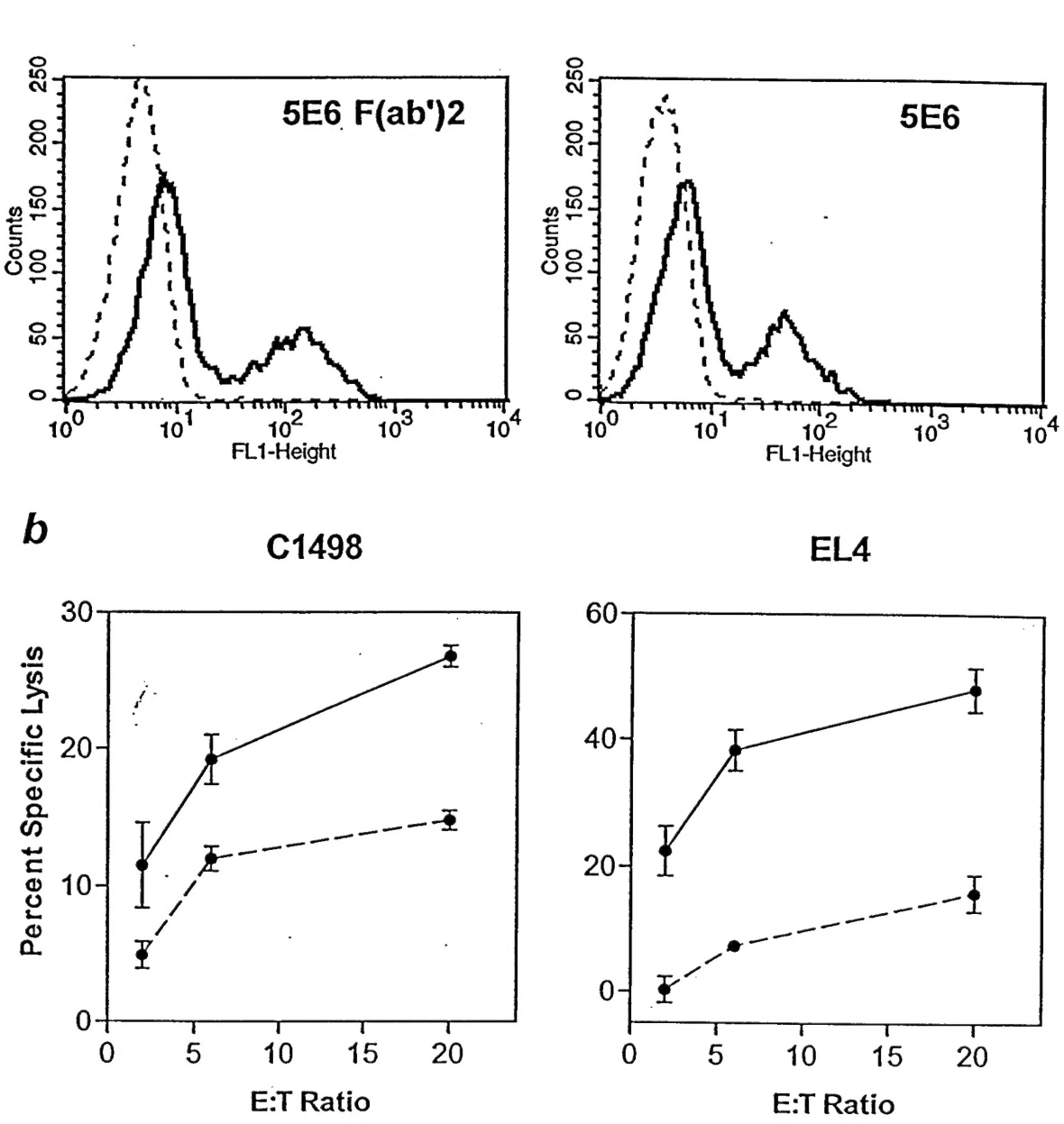
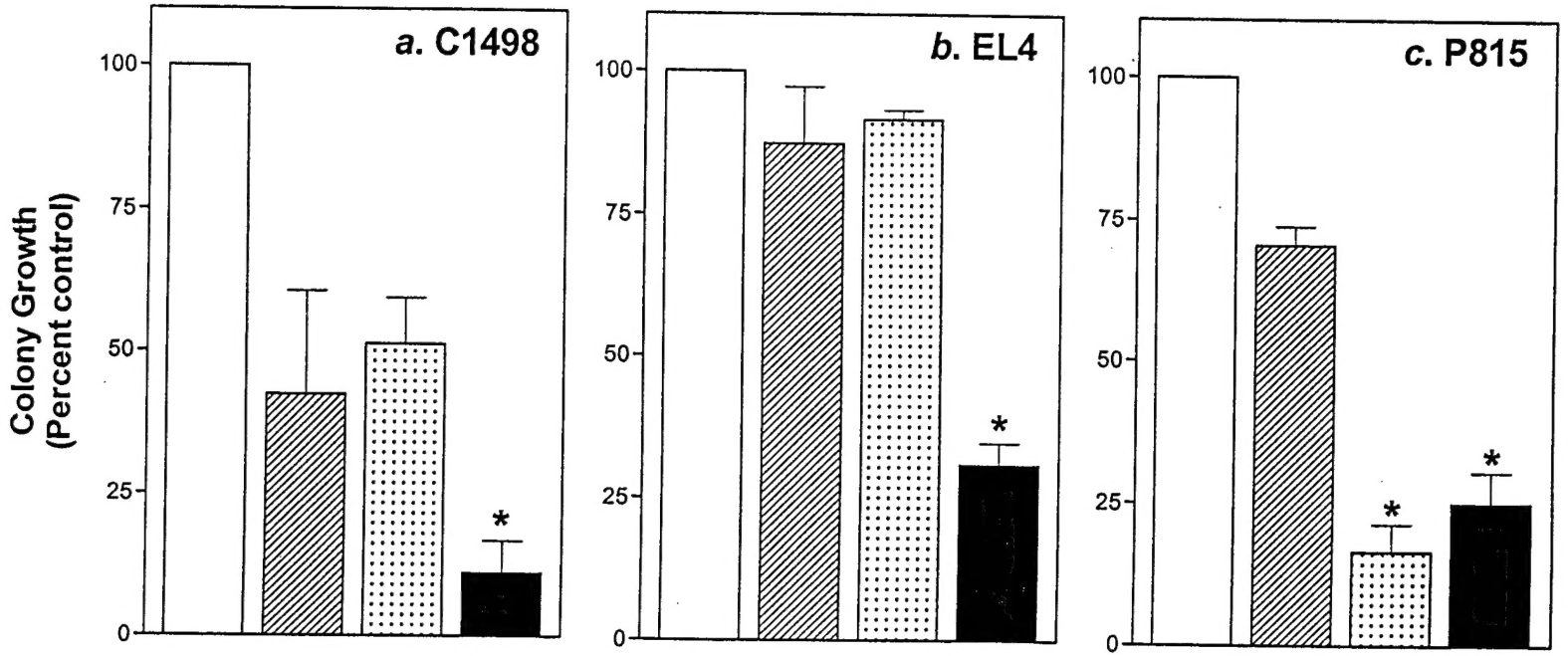


Figure 1. Flow cytometry and cytotoxicity assays of IL-2 activated purified B6 NK cells.
a. Staining with unlabeled F(ab')$_2$ 5E6 mAbs (solid line) or with normal mouse IgG (dashed line), followed by FITC-labeled goat anti-mouse IgG F(ab')$_2$ (left). Staining with biotinylated whole 5E6 mAb (solid line) or not (dashed line), followed by FITC-streptavidin (right). Both reagents stained about 30% of the NK cells. Y-axis, cell number; X-axis, mean fluourescence intensity.
b. Lysis of ^{51}Cr-labeled syngeneic C1498 or EL-4 tumor cells by sorted NK1.1$^+$ 5E6$^+$ NK cells pretreated for 60 min. with normal mouse IgG (dashed lines) or 5E6 F(ab')$_2$ mAb (solid lines) over a 4 hour period. Y-axis, mean and SEM specific lysis, X-axis, effector:target cell rato. At each point, the mean values between control and F(ab')2 were signficantly different ($p < 0.05$).

Figure 2. Treatment of NK cells with 5E6 F(ab')$_2$ results in decreased tumor growth in vitro. Various numbers of B6 SCID NK cells activated with rhIL-2 for 5-7 days were plated in U-bottom 96 well microtiter plates at 50 µl/well and pre-treated with medium alone or 25 µg/ml F(ab')$_2$ fragments of normal mouse IgG (NMG, hatched bars), 5E6 anti-Ly49 I & C (solid bars), or 4D11 anti-Ly49G2 (dotted bars) for 3 h at 37 C. 100, 50 or 25 C1498, EL-4 or P815 cells, respectively, were added at 50 µl/well and the cells were co-cultured for 48 h. As control, tumor cells were cultured alone (open bars). The cells were transferred into a semi-solid matrix and cultured for 5-7 days to detect tumor colonies. C1498 H2^b at NK:tumor ratio of 2:1 (a) EL-4 H2^b at NK:tumor ratio of 10:1 (b) P815 H2^d at NK:tumor ratio 100:1. Data from a representative of 3 experiments are shown, mean % control where control = 100%. The stars indicate significantly lower values than NMG, $p < 0.05$, by Student's t test.



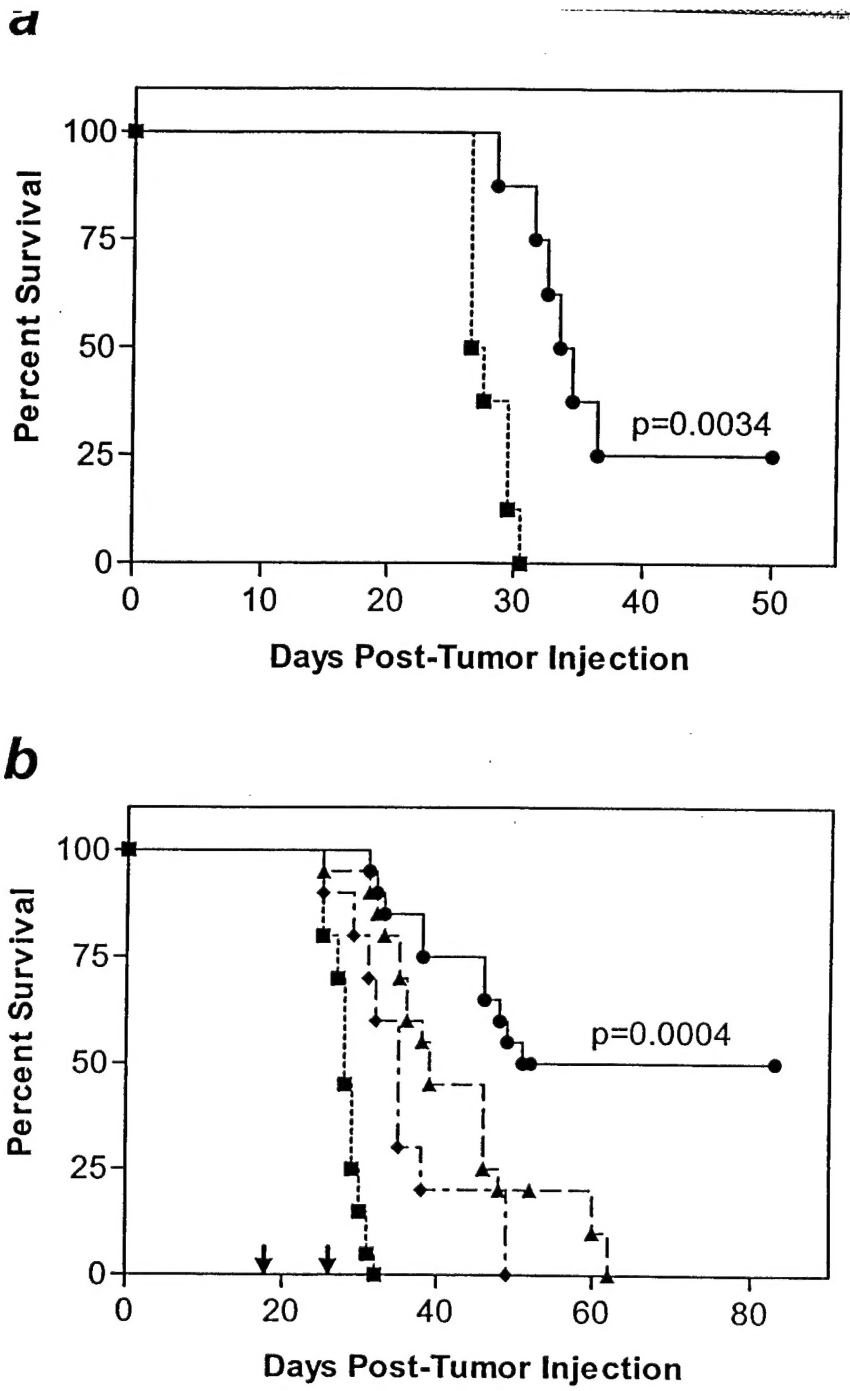


Figure 3. 5E6 F(ab')$_2$ treatment protects B6 mice against C1498 leukemia cells. a. Mice received 180μg 5E6 F(ab')$_2$ (circles) or PBS (squares) i.p. 2 days before and twice weekly for 3 weeks after infusion of 3 X 10^4 C1498 cells. This is a representative of 4 experiments (n = 8 for each group of each experiment. b. 12 x 10^6 IL-2 activated B6 NK cells were incubated with 300 μg 5E6 (circles) or 4D11 (diamonds) F(ab')$_2$ or 5% normal mouse serum (NMS) (triangles) for 2 h at 37 C and further co-cultured with 1.2 X 10^6 C1498 cells for 25 h. As controls, 1.2 X 10^6 C1498 cells were cultured alone (squares). After culture mice received 10^5 C1498 cells and 10^6 NK cells from the cultures i.v. At days 18 and 25 (arrows) mice were infused with inocula of 5 X 10^6 NK cells treated with 5E6 or 4D11 F(ab')$_2$ or NMS ex-vivo followed by IL-2 injection (5 X 10^4 IU i.p.) for 3 days. Pooled data form 2 independent experiments (n = 20/group except for 4D11, n = 10) are shown. The p values were determined by Log-Rank test.

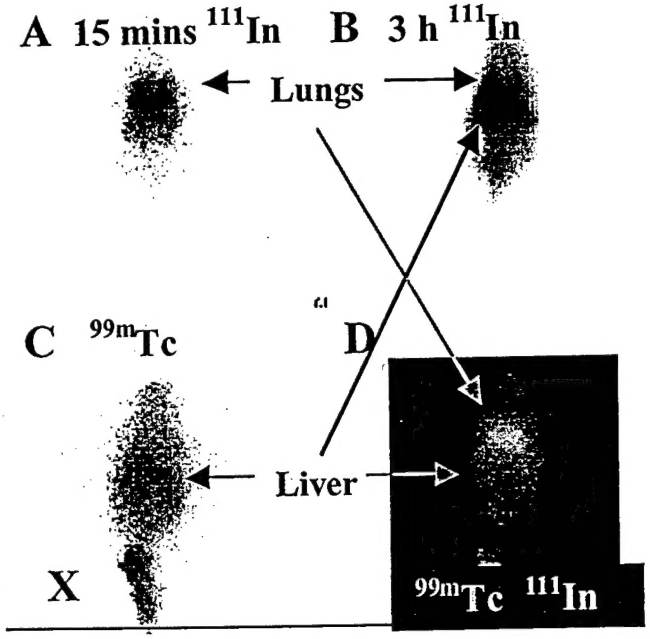


Fig. 4. Images of ^{111}In-labeled (220 µCi) YAC-1 lymphoma cells (6 million) infused IV into tail vein of a BALB.NK1.1 mouse. **A** 15 mins post IV (ROI showed 27 kcounts from lungs) **B** Same mouse after 3 h (ROI showed 10 kcounts from lungs (38%) and 16 kcounts for liver/spleen). **C** ^{99m}Tc agent was infused just before 3 h measurement and detected from second channel showing localization to liver. **D** Overlay of ^{99m}Tc and ^{111}In channels. Initially cells were localized to the lungs. Later image revealed decreased radioactivity in the lungs, with accumulation of the isotope in the liver and spleen. Post mortem *ex vivo* showed ratio of lungs to liver as 46%. Thus, *in vivo* imaging provided closely similar estimate to traditional *ex vivo* analysis with potential for long-term dynamic studies

Key research accomplishments

* Blocking negative signals to NK cell Ly49 receptors augments lysis and inhibiltionof growth of tumor cells in vitro and in vivo

* Blocking negative signals with non-depleting F(ab')2 anti-Ly49I & C mAbs increases survival of mice infused with lethal doses of syngeneic leukemia cells

Reportable outcomes

- manuscript in review by *Blood* journal
- patent for this approach has been designed
-funding has been applied for based on this work

Conclusions

The ability to block inhibitory signals to NK cells from class I antigens expressed by tumor cells allows more effective anti-leukemia function of NK cells against syngeneic tumors. We recommend that we extend these studies to the use of more $F(ab')_2$ mAbs to inhibitory receptors and to eventually test this approach with human tumors and blocking antibodies to the human NK cell inhibitory receptors by the same type of in vitro methods, and use NOD-SCID mice as hosts of HLA typed tumor cells and HLA type purified $CD56^+$ NK cells.

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Appendices: none